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Effect of High-Fat Diet on the Regulation of Vascular Tone in Arteries of Young Mice

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Abstract

The prevalence of overweight and obesity is increasing in all age groups and continents. The leading cause is nutritional intake of high amounts of fat. Obesity is a major risk factor for endocrine and cardiovascular diseases like high blood pressure, insulin resistance and the subsequent development of atherosclerosis. Childhood obesity affects cardiac function instantly and is a risk factor for coronary heart disease and unfavorable metabolic changes in adulthood, independent from body weight at that time. The objective of this study was to investigate changes in vascular reactivity of the aorta and carotid arteries of young mice in response to high dietary fat intake. 4 weeks old C57BL/6J mice were either fed a high-fat diet (Obese group) or standard rodent chow (Control group) for 30 weeks. Vascular responses to phenylephrine (PE) and acetylcholine (ACh) were investigated in aortic and carotid artery rings of these animals *ex vivo,* in absence and presence of nitric oxide (NO) and endoperoxides. Compared to the Control group, the mice of the Obese group showed glucose intolerance, greater weight gain and altered responses to vasoactive agents. The contractile response to PE was enhanced and was further augmented by inhibition of NO production in the aorta of the Obese group, whereas in the carotid artery of the Obese group and in the Control group the contraction remained unaffected. Relaxations to ACh were not diminished in the Obese group. In untreated vessels, contractions to high concentrations of ACh were observed in the carotid artery. They were abolished by an unspecific cyclooxygenase-inhibitor. Inhibition of endogenous NO production abrogated endothelium-dependent relaxations to ACh and contractile responses were observed in all vessels. These endothelium-dependent contractions were stronger and started at lower concentrations in the carotid artery and were increased by obesity. In conclusion, the findings of the present study suggest that a high dietary fat intake leads to weight gain, glucose intolerance and enhanced vascular contractile response in adolescent mice. The heterogenous diet-induced changes indicate selective effects on different vascular beds. These findings may be important to understand the variability in the onset and progression of vascular diseases in different vascular beds in obese patients and to develop efficient therapeutic strategies.

List of Abbreviations

ACh	Acetylcholine
AT_{1B}	Angiotensin Receptor, subgroup 1B
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BMI	Body Mass Index
cGMP	Cyclic Guanosine Monophosphate
COX	Cyclooxygenase, subgroups COX-1, COX-2
DAG	Diacylglycerol
EDCF	Endothelium-Derived Contracting Factor
EDHF	Endothelium-Derived Hyperpolarization Factor
eNOS	endothelial Nitric Oxide Synthase
ET_A	Endothelin Receptor, subgroup A
HFD	High-Fat Diet (57% kcal of lipids)
iNOS	inflammatory Nitric Oxide Synthase
IP_3	Inositol Triphosphate
KCl	Potassium Chloride
L-NAME	N^G-Nitro-L-Arginine Methylester
M_3	Muscarinic Receptor, subgroup 3
Meclo	Meclofenamate
nNOS	neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase (subgroups eNOS, nNOS, iNOS)
PE	Phenylephrine
PIP_2	Phosphatidyl-Inositol-Biphosphate
PKC	Proteinkinase C
PPARγ	Peroxisome Proliferator-Activated Receptor
ROS	Reactive Oxygen Species
WHO	World Health Organisation

1. Introduction

1.1. Obesity

1.1.1. Prevalence

Obesity is a disease affecting a large number of people and has become an important healthcare issue over the last few decades. It is defined as a body mass index (BMI) being greater than 30,[1] while the range between 25 and 30 counts as overweight. The prevalence of overweight and obesity is still increasing and affects all age levels in developed as well as in developing countries.[2-6] Instead of using overweight and related body mass index (BMI) as indicators for the prognosis of pathological changes, recent studies emphasize that the distribution of the body fat is crucial for accurate estimations. In this context the term of visceral obesity measured by the waist-circumference has been coined and is used as a prediction value.[7] Like in the rest of Europe, the number of individuals being overweight or obese is increasing in Switzerland.[8,9] National data from 1999 shows a prevalence of overweight and obesity of 21.8% and 4.5% for women and 39.2% and 5.8% for men.[10] For both combined, an increase from 44% to 59% for men and from 24% to 37% for women was reported in 2005.[8] Even though prevention campaigns have been launched in several countries, prognoses for the prevalence indicate clear upward trends for overweight and obesity in the future.[10-12] The prevalence of cardiovascular disease as a consequence of obesity is expected to increase by 5%, leading to an estimate of 16%, by 2020.[13]

1.1.2. Childhood Obesity

Obesity in childhood is becoming increasingly prevalent.[14] The presence of obesity or the related metabolic syndrome at young age is a significant predictor of cardiovascular disease and other health problems in adulthood.[15-17] Long-term consequences of pediatric obesity are independent from body weight as an adult and include higher risk for early death.[18,19] Like in adulthood, overweight in childhood is related to various pathologic states such as alterations in endothelium-

dependent dilation and increased intima-media thickening.[20,21] High blood pressure, impaired glucose tolerance, insulin resistance and endothelial dysfunction can be observed in arteries of overweight children and adolescents and similarly in animals fed a high-fat diet.[20-23] Although functional or macroscopic vascular changes in obese children are mostly asymptomatic, they can affect cardiac function in children.[24,25] Besides the vasculature, other organs and systems can be affected and orthopedic, neurologic, pulmonary and gastroenterologic problems can arise.[18,25,26] To protect children efficiently from these obesity-associated health problems, it is important to improve the efficacy preventory measures.[27]

1.1.3. Consequences of Obesity

1.1.3.1. Metabolic Syndrome

Obesity is a complex disorder, genetic and environmental factors contributing to its development.[28,29] Since the genetic background of the human race seems not to have changed dramatically over the last few decades, factors like decreased physical activity and excessive nutrition are suspected to play a crucial role.[30] It is associated with other cardiovascular risk factors, such as insulin resistance and subsequent development of type II diabetes.[31] Other diseases associated with overweight and obesity are high blood pressure, high cholesterol levels and dyslipidemia, altogether forming a cluster of symptoms known as the metabolic syndrome.[4,32,33] The syndrome has been the topic of numerous studies since it was established in 1977 and definitions for the diagnosis of the metabolic syndrome have been adapted by various organizations, including the World Health Organization (WHO).[32,34] It is widely accepted that the metabolic syndrome is associated with a significantly elevated cardiovascular and metabolic risk.[35,36] Cardiovascular disease not only affects the quality of life and morbidity, but is the principal cause of death in the United States of America, Europe, and major parts of Asia.[37]

1.1.3.2. Economic Aspects

The treatment of obesity and the associated cardiovascular diseases are responsible for estimated 2 to 6.8% of the health care expenses and thus is a heavy burden for the health care system.[38] These data do not only represent the situation in Canada and the USA, where the prevalence of overweight and obesity is generally higher than in European countries, but the high impact has similarly been reported for Switzerland.[38-40] In line with the growing prevalence of obesity, the economic impact is expected to increase dramatically in the future.[12,39]

1.1.3.3. Effect of Nutritional Change on Vascular Function

Obesity as well as atherosclerosis and hypertension are defined as chronic diseases.[41] Various risk factors such as overweight,[42] cigarette smoking,[43] diabetes mellitus,[44] high blood pressure and elevated blood lipid levels[45] lead to pathologic changes in the endothelium's major functions like the homeostasis of blood flow and the secretion of tone- and coagulation-modulating agents. They are related to each other as obesity can cause changes in vascular function and, as a macroscopic equivalent, atherosclerotic lesions can occur. Changes on molecular level are multifaceted and precede macroscopic, sonographic and even microscopic changes of atherosclerosis.[46] Metastudies show that a diet containing high amounts of fat increases the likelihood of gain in body mass in rodents and humans.[47] The outweighing part (89%) of this weight gain is due to fat accretion.[48] In 1983, Anitschkow and Chalatow in a seminal work have shown that animals (rabbits and guinea pigs) fed with egg yolk developed hypertrophy of the intima and infiltration of fatty substances in the aorta.[49] In the subsequent series of experiments, they extracted cholesterol as the responsible agent.[49] This substantiates the hypothesis that not only the amount of fat but also its biochemical origin influences the consequences on vasculature.[50] Although the composition of dietary fat can influence the severity of weight gain and associated vascular changes, it is reported that the amount of dietary fat is of higher importance.[50] Increased dietary fat intake induces insulin resistance,[51] dyslipidemia and hyperleptinemia,[52] salt sensitivity,[53] increased oxidative stress levels[54] and high

blood pressure.[53] These morbidities lead to endothelial dysfunction and enhance vasoconstriction.[55] The development of atherosclerosis as a complex disorder was first formulated as the hypothesis of "response-to-injury".[56] The model was adapted later leading to the characterization of atherosclerosis as an inflammatory disease.[57] Large prospective studies showed that the extent of atherosclerotic lesions in the coronary arteries correlates with the grade of obesity in men.[24,58]

1.1.3.4. Role of Vasoactive Molecules

The endothelium plays an important role in the regulation of blood flow and metabolic processes taking place in the vascular wall.[59] By synthesizing and secreting various vasoactive substances, endothelial cells are involved in controlling the tone of smooth muscle cells, platelet aggregation, leukocyte migration, inflammatory processes and response to circulating substances.[60-62] Modulation of the diameter of the vessel is the result of a complex interaction between vasoconstricting and vasodilating agents. This balance is disturbed in obesity and vasoconstriction is enhanced. Some of the factors involved in the regulation are released by the endothelium itself, others are delivered by the blood flow. The most important vasoconstrictors are endothelin-1,[63] angiotensin II,[64] thromboxane A_2[65] and α-adrenergic ligands like epinephrine. In obesity, besides an increase in the synthesis of molecules effecting contraction, a parallel increase in the expression of the interacting receptor such as angiotensin- (AT_{1B}) and endothelin-receptors (ET_A) is observed.[21] Furthermore, high-fat diet increases the level of reactive oxygen species (ROS).[66] These molecules can directly inactivate nitric oxide (NO), thereby inhibiting vasodilation.[54,67] High levels of free fatty acids observed in obesity alter the vascular reactivity by enhancing α_1 adrenoceptor-mediated contractions in vascular smooth muscle cells.[68] Under certain circumstances, Acetylcholine (ACh) has vasoconstrictor effects generating endothelium-derived contracting factors (EDCFs) via the action of cyclooxygenase (COX). Vasodilatory factors include prostacycline (prostaglandine I_2),[69] ACh,[59] NO,[70] bradykinin[71] and the endothelium-derived hyperpolarization factor (EDHF).[72] Prostacycline leads to vasodilation by activating adenosine triphosphate (ATP)-sensitive potassium

channels.[73] ACh activates endothelium-dependent NO-synthesis[74] and bradykinin leads to the formation of various vasodilating factors including NO, EDHF and prostanoids. Although the existence of an EDHF has been demonstrated in different arteries and species, the physical equivalent is still under investigation. Factors that probably play a role are potassium ions, hydrogen peroxide and epoxyeicosatrienoic acids.[75] Besides these vasoactive substances, several growth factors and enzymes like proteases play a role in the vascular remodeling leading to atherosclerosis.[76]

1.1.4. Vasoactive Agents

1.1.4.1. Effects of Phenylephrine

Phenylephrine (PE) is a synthetic drug derived from epinephrine. Besides the 3-hydroxy-group, it is structurally identical with epinephrine, the sympato-mimetic endogenous stress-hormone secreted by the adrenal gland.[77] PE is a selective agonist on adrenergic α-receptors.[78,79] The group of adrenergic receptors can be divided into two main groups, α (α_1, α_2) and β (β_1, β_2, β_3).[80,81] The expression pattern, intracellular pathways and thus downstream effects differ between the subtypes.[82] One important action of adrenergic receptors is modulating vessel tone and thereby systemic blood pressure. While activation of α_1- and β_2-receptors mediates vasoconstriction (α_1 via phospholipase C [PLC], β_2 by inactivating adenylyl-cyclase), binding to α_2-receptors (activating adenylyl-cyclase) leads to vasodilation.[83] The activation of the G-protein coupled α_1-receptors activates PLC, which leads to the cleavage of phosphatidyl-inositol-biphosphate (PIP_2) into the second messengers inositol-triphosphate (IP_3) and diacylglycerol (DAG).[84] IP_3 effectuates the release of calcium from the sarcoplasmatic reticulum and activation of proteinkinase C (PKC), causing depolarisation and therefore contraction of the vascular smooth muscle cell.[85] This pathway is known as the phosphatidyl-inositol-pathway.

The contractile response of the smooth muscle cells following the exposure to PE occurs in healthy as well as in endothelium-denudated arteries and is therefore endothelium-independent.[86]

Endothelial denudation can even increase the vasoconstrictor reaction up to two-fold.[87] This finding can be explained by the localisation of PE-receptors directly on smooth muscle cells as well as on the cell membrane of endothelial cells, where they have inverse effects on the vascular tone by causing vasodilation.[88] Furthermore, the PE-mediated effects can be modulated by the expression of its receptors.[21]

1.1.4.2. Effects of Acetylcholine

Vasodilation in response to ACh is endothelium-dependent and is mediated by NO, former known as the endothelium derived relaxation factor.[62,70] If the layer of endothelial cells is removed, dilatory responses are abolished and ACh leads to vasoconstriction.[89] By binding on a muscarinic receptor (M_3) located on the endothelial wall, ACh induces complex intracellular mechanisms. The phosphatidyl-inositol-pathway is activated and triggers calcium-release and activation of calmodulin, which binds to the endothelial NO-synthase (eNOS). Binding of calmodulin results in the synthesis of the vasodilating agent NO.[90] The enzyme NO-synthase (NOS) converts L-arginine, a semi-essential proteinogenic amino-acid, to NO and citrulline.[91] As NO is gasiform, it diffuses through the cell membrane of the endothelial cell into the smooth muscle cell can there activate the soluble guanyl cyclase. The subsequent production of cyclic guanosine-monophosphate (cGMP) activates cGMP-dependent protein kinases which effect most of the intracellular actions modulating the conductance of potassium channels, leading to hyperpolarisation and finally to relaxation of smooth muscle cells.[92] Preincubation with L-nitro arginine methylester (L-NAME) abolishes the dilatory response by blocking the NOS. Genetic knock-out of the eNOS has the same effect ($eNOS^{-/-}$ mice).[93-95] Besides eNOS, two other isoforms of the enzyme located in various tissues are known, the neuronal NOS (nNOS) and the inducible inflammatory NOS (iNOS).[74,96] Under certain circumstances, ACh leads to endothelium-dependent vasoconstriction. The degree of contraction depends on the concentration of ACh (onset from 10^{-7} mmol/L). Since the exact mechanism of contraction is still unsolved, the term of the "endothelium-derived contraction factor" (EDCF) was

introduced.[97] Subsequent research on the subject proposed several endoperoxide molecules like prostaglandin H_2,[95] thromboxane A_2,[98] prostaglandin I_2,[99] prostacyclin[100] and isoprostanes[101] as effectors. Endoperoxides are synthesized by COX and cause vasoconstriction. They can be further transformed by specific synthases into molecules like thromboxane, prostaglandins or prostacyclin.[102] The activation of COX in the endothelium must thus play a key role in the process of modified ACh-responsiveness in obesity,[103,104] as its inhibition with non-selective COX-inhibitors (indomethacin or meclofenamate [Meclo]) abolishes the response.[95,105] Knocking out the COX-1 gene abolishes EDCF-dependent responses, whereas knocking out the COX-2 or its chemical inhibition does not block contractile effects.[106,107] Thus in mice, COX-1 seems to play a crucial role.[107] Prostacyclin synthase is upregulated in hypertensive and aged animals[108] and causes an increased vasoconstriction in these animals.[100] The role of thromboxane A_2 remains unclear, as subsequent studies have shown that thromboxane A_2 synthase inhibitor does not affect the EDCF response to ACh or even that the addition of thromboxane A_2 acts dilatory and abolishes the contractile response.[100] It was also observed that vasoconstriction in arteries induced by a high-cholesterol diet can be abolished by a thromboxan A_2 receptor antagonist and that contractile responses to ACh can be blocked by antagonizing the thromboxan-prostaglandin-receptor or more specifically antagonizing the receptor of prostaglandin H_2 on the smooth muscle cell layer.[95,103,109]

1.1.5. Heterogeneity of Vascular Beds

Arteries can be divided in elastic and muscular type based on their microscopic architecture. Whereas in elastic type arteries the tunica media contains a large percentage of elastic fibres, in muscular type arteries it consists predominantly of smooth muscle fibers. The arteries also show differences on molecular level, as in the elastic arteries, vimentin is the major smooth muscle cell filament protein, whereas in the muscular arteries both vimentin and desmin are found.[110,111] The aorta and its direct branches build the group of the elastic type arteries, the more distally located conduct vessels are from the muscular type. The aorta and carotid arteries are typical representatives of elastic type arteries, whereas the femoral artery belongs to the group of muscular arteries. Both groups are though susceptible to atherosclerosis and stiffening with aging.[112-114] They however differ in their lumen diameter and in the thickness of vascular smooth muscle cell layer. The extent of atherosclerotic plaques in the aorta and carotid artery correlates with the extent of coronary artery disease.[115] Despite these similarities, the impact of the vasomodulator factors differs between the vascular beds. The carotid artery is more sensitive to ACh than the femoral artery in young C57BL/6J.[116] In the carotid artery, the activation of the cGMP-dependent NO-pathway by ACh is predominantly responsible for relaxations, whereas in the femoral artery a K-channel-dependent pathway is involved.[116] In young C57BL/6J mice ACh leads to contractions in the carotid artery, but not in the femoral artery.[116] In $eNOS^{-/-}$ mice, ACh leads to contractions not only in the aorta, but also in femoral and carotid arteries.[95] Interestingly, in humans, the aorta seems to be more susceptible to atherosclerotic changes than the carotid artery.[117,118]

1.2. Aim of the Study

Obesity is a disease, which has developed epidemic character and thus has become an important issue in health care and research. The prevalence of overweight and obesity is rapidly increasing not only in adults, but also in children. Obesity in childhood augments the risk of cardiovascular morbidity in adulthood. The underlying mechanisms of obesity-mediated changes of vascular reactivity have been investigated extensively, but new findings posed new questions, and several remain unsolved to this day.

Given the increasing importance of obesity in youth, the present study was conducted *ex vivo* using aorta and carotid artery from young C57BL/6J mice, an approved rodent model for diet-induced obesity. The aim was to investigate, if short-term high-fat diet feeding induces changes in vascular reactivity in young mice, and how reaction patterns to different vasoactive agonists vary between diet groups and vascular beds.

Main topics of interest were:

1. The effects of a high dietary fat intake on vascular reactivity in different vascular beds (aorta and carotid artery) of young mice
2. The impact of NO and prostanoids on vascular responsiveness after high-fat diet

2. Methods and Materials

2.1. Animals

The study was conducted using healthy male mice (C57BL/6J, Charles River, Sulzfeld, Germany). Animals were kept at the animal facility of the "Biologisches Zentrallabor" of the University Hospital of Zurich. In addition to specified diets, mice were provided tap water *ad libitum*. The animals were exposed to twelve hour light/dark cycle. The room temperature was maintained at 22°C. Experimental setup and animal accommodation were approved by the "Kommission für Tierversuche des Kantons Zürich" Switzerland, the local authority for animal research, and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Dietary Treatments

Mice were either fed a standard chow (12.3% of total kcal from fat, Kliba Nafag 3430, Kaiseraugst, Switzerland, referred as Control group) or a high-fat diet (58% of total kcal from fat, Research Diets D12331, referred as Obese group). The macronutrient compositions of the diets are reported in **Table 1**. The major source of the lipid fraction of the high-fat diet was derived from coconut oil, mostly containing saturated fatty acids (90%).[119]

Table 1 Major macronutrient constituents of diets given in percent of kcal

Diet	**Standard Chow**	**High Fat**
Protein	22.4	16.4
Carbohydrate	65.4	25.5
Fat	12.3	58

2.3. Groups

Healthy male C57BL/6J mice of 4 weeks of age were randomly assigned to one of the following groups:

- Control: Fed with standard rodent chow for 30 weeks
- Obese: Fed with high-fat diet for 30 weeks

2.4. Glucose Tolerance Test

The animals were starved overnight, body weight was measured and venous blood was obtained from the tail vein (0 min) for baseline glucose measurement. An AccuChek Advantage glucose meter (Roche Diagnostics, Switzerland) was used for this purpose. Blood samples were taken at 5, 10, 15, 30, 45, 60, 90, and 120 min after the intraperitoneal injection of D-glucose (2 mg/g body weight).

2.5. Preparation of the Arteries

On the day of the experiment, mice were anesthesized with xylazine (100 mg/kg body weight [BW]), ketamine (23 mg/kg BW) and acepromazine (3.0 mg/kg BW), all intraperitoneal. When pain reflexes (in response to strong pain triggers on the toes) were absent, abdomen and chest were opened by laparotomy and medial sternotomy. Subsequently, exsanguination via cardiac puncture was performed. Organs were removed and immediately frozen in liquid nitrogen for further investigation. Blood vessels were identified, carefully excised and placed in cold (4°C) Krebs-Ringer bicarbonate solution of pH 7.4. Composition of Krebs buffer (in mmol/L): 118.6 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25.1 $NaHCO_3$, 1.2 KH_2PO_4, 0.026 $EDTA_{Na2Ca}$ and 10.1 glucose. Under a microscope (Olympus SZX9, Volketswil, Switzerland), adherent connective, fat and muscle tissue was removed carefully from the arteries with microsurgical instruments. Special care was taken to prevent endothelial damage or disturbance of the integrity of the smooth muscle cells during this procedure. Vessels were then cut into rings (thoracic aorta 3 mm, carotid artery 2.0 - 2.5 mm).

2.6. Vascular Function Experiments

2.6.1. Organ Chambers

Vascular rings were mounted onto two tungsten hooks (100 μm diameter) under the microscope. The aortic and carotid artery rings were transferred to the water-jacketed pre-warmed organ chambers containing aerated (95% O_2, 5% CO_2) Krebs solutions (pH 7.4, 37°C). Chamber volume was 10 ml. The rings were connected to a force transducer (Hugo Sachs, Mach-Hugstetten, Germany) that allowed recording of isometric tension via an anchor. Before stretching the vessels, an equilibration time of 30 minutes was provided. To achieve an optimal level of passive tension, vessels were stretched in a stepwise manner. Optimal stretching weights for aorta and carotid artery rings (as investigated in earlier experiments in the laboratory) were for aorta 2.5 g and for carotid artery 1.75g. After another equilibration period of 20 minutes, the integrity of the vascular smooth muscle cell layer was verified by repeated exposure to potassium chloride (KCl, 100 mmol/L, iso-osmotically replaced), until a stable response was achieved. Recordings were transferred to an X/Y-plotter (Rikadenki Electronics, Tokyo, Japan) and printed.

2.6.2. Experimental Protocols

2.6.2.1. Phenylephrine

After verifying the integrity of the layer of smooth muscle cells with KCl as described above, the aortic and carotid artery rings were thoroughly washed with Krebs buffer. Vascular rings were either left untreated or L-NAME (300 μmol/L) was added to the solution in the organ chamber and incubated for 30 minutes. Subsequently, PE, an adrenergic α_1-receptor agonist, was added in cumulative concentrations starting at 0.1 nmol/L and titrated to 50% KCl.

2.6.2.2. Acetylcholine

To measure responses to ACh, vessels were either left untreated, or incubated with the non-selective cyclooxygenase inhibitor Meclo (1 µmol/L) or L-NAME (300 µmol/L). After 30 minutes of incubation, vessels were precontracted to 50% of the KCl response by using cumulating concentrations of PE. Endothelium-dependent response to ACh (0.1 nmol/L - 300 µmol/L) was then examined by adding the agonist in a stepwise manner as shown in **Fig. 1**.

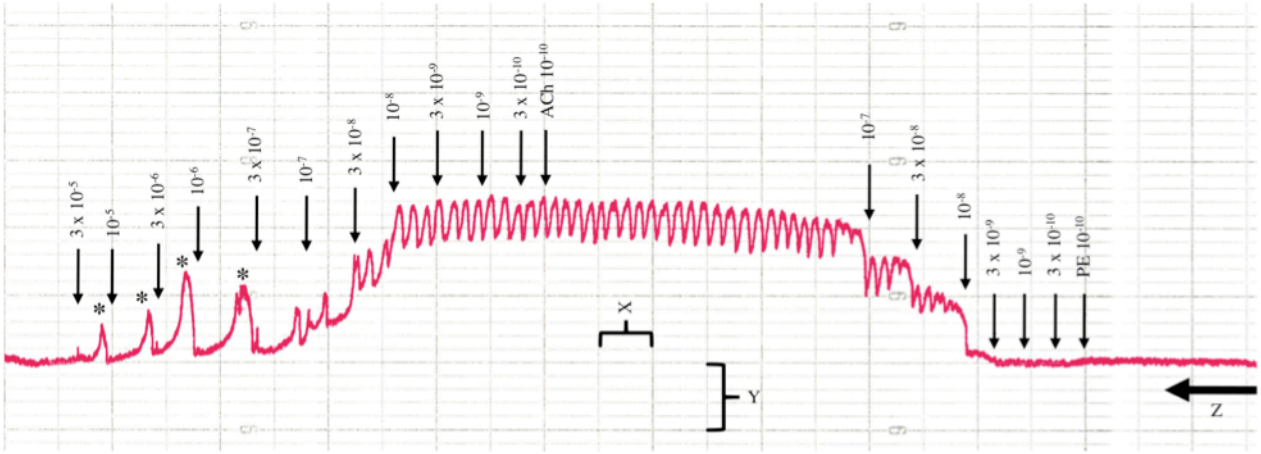

Fig. 1: Representation of a typical contraction and relaxation curve of a carotid artery. X correlates to 5 minutes, Y correlates to 0.25g. Z indicates the direction of the protocol, starting with phenylephrine 0.1 nM (10^{-10} mol/l). * indicate endothelium-dependent contractions to ACh.

2.7. Drugs

Acepromazine (Fatro, Ozzano Emilia, Italy), xylazine (Bayer, Zurich, Switzerland) and ketamine (Chassot AG, Bern, Switzerland) were used for anesthesia. Chemicals used for vascular function experiments were ACh, PE, Meclo, all obtained from Sigma-Aldrich (Buchs, Switzerland), and L-NAME (obtained from ALEXIS Biochemicals, Lausen, Switzerland). Drugs were dissolved in purified water (Millipore®, Volketswil, Switzerland) and diluted with fresh cold Krebs solution to attain the concentration needed. Concentrations are expressed as final molar concentration in the organ chambers.

2.8. Calculations and Statistical Analysis

The printed charts were manually digitized. The peak of the vascular response to the second exposure of potassium chloride (KCl, 100 mmol/L) was taken as reference value for contractions. Relaxations were expressed as percentage of precontraction in response to PE.

For statistical analysis Microsoft Excel® for Windows® version 2003, StatView® for Windows® version 5.0.1., FitLab® and Prism Graph Pad® version 4.0 for Windows® were used. A *P* value smaller than 0.05 was considered significant and "n" equals the number of animals, that were used for analysis. Two-tailed paired or unpaired Student's *t*-test, ANOVA for repeated measurements followed by Bonferroni's correction, calculations of the AUC and half maximal effective concentration (EC_{50}) or pD2 (=-log[EC_{50}]) were used when appropriate for the dataset. The paired t-test was used to compare data within the same vessel and treatment group. Otherwise, the unpaired t-test was used. ANOVA was used to compare reactions to cumulating concentrations of ACh at different time points, as were AUC and pD2 as well.

Values were represented in bar-graphs, line-graphs or tables. To emphasize the difference between relaxation and contraction, percentages for relaxations were shown as negative values, whereas percentages for contractions were displayed positively. The intervals of the scales were adjusted according to the values of the results.

3. Results

3.1. Effect of High-Fat Diet on Weight Gain

The changes in body weight were monitored throughout the experimental feeding period of 30 weeks. In **Table 2**, the absolute weight gain in the Control and Obese group is listed. The gain in weight was calculated by subtracting the initial weight, measured before starting the diet, from the final weight at 34 weeks of age. Mice of the obese group gained significantly more weight than those of the control group of the same age after 30 weeks of high-fat diet.

Weight Gain

Group	Control	Obese
Weight gain (g)	13.8 ± 0.7	16.4 ± 0.4*****

Table 2: Weight gain of mice fed with standard rodent chow or high-fat diet for 30 weeks.

* *P*<0.05 vs. Control. Control: n=10, Obese: n=7. Values represent means ± standard error.

3.2. Effect of High-Fat Diet on Glucose Levels

Blood glucose levels were measured in starved Control and Obese animals and at 5, 10, 15, 30, 45, 60, 90 and 120 min after intraperitoneal injection of D-glucose (2mg/g body weight), **Fig. 2**. Before the injection, glucose levels were similar. In the Obese group, glucose levels raised markedly higher in comparison to the Control group (significant by ANOVA-analysis $p<0.05$ and the student *t*-test of the AUC values [below in **Table 3**], final values at 120 min; Control: 7.7±0.4 mmol/L, Obese: 10.1±0.5 mmol/L). This difference suggests impaired glucose tolerance.

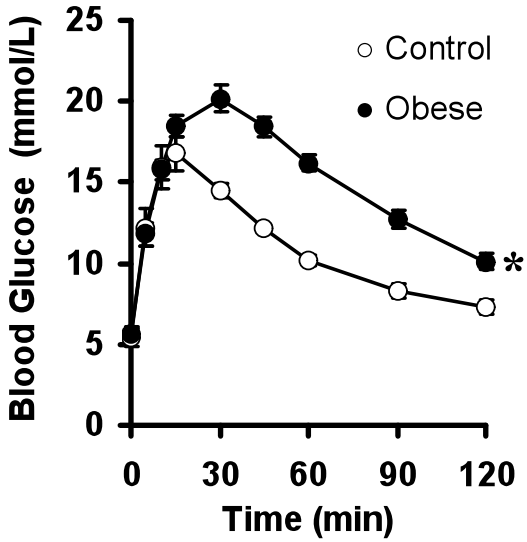

Fig. 2: Effect of high-fat diet on blood glucose levels at 0 to 120 min after intraperitoneal injection of D-glucose (2 mg/g body weight) in mice fed a standard chow (Control) or high-fat diet (Obese) for 30 weeks. Control: n=6, Obese: n=16. Values represent means ± standard error. * *P*<0.05 vs. Control compared by ANOVA, and AUC compared by unpaired students *t*-test. Values for AUC are represented below.

Area Under the Curve of Blood Glucose Levels after Intraperitoneal D-Glucose Injection in Control and Obese Mice

AUC	
Control	**Obese**
1308.8± 31.6	1814.1 ± 53.1*

Table 3: AUC values for blood glucose levels in response to D-Glucose injection (2mg/g body weight) in mice of the Control and Obese group. Values represent means ± standard error and are given in arbitrary units. * *P*<0.05 vs. Control. Values were compared by unpaired student's *t*-test.

3.3. Effects of High-Fat Diet on Vascular Function

3.3.1. Effect of High-Fat Diet on Response to Phenylephrine

Treatment with high-fat diet markedly increased PE-dependent contraction in the aorta and carotid artery, **Fig. 3A** and **3B**. In the aorta, high-fat diet induced a 14-fold increase in contraction (Control: 2.6 ± 0.7%; Obese: 35.9 ± 6.5%), **Fig. 3A**, whereas in the carotid artery, a 16-fold increase was observed (Control: 3.3 ± 1.6%; Obese: 53.0 ± 6.9%), **Fig. 3B**. Additionally, vessels were treated with L-NAME to investigate vascular responsiveness in NO-depleted conditions. In the aorta, the PE-induced contractile response was further enhanced in the Obese group (1.8-fold versus Untreated; 68.0 ± 8.2% vs. 35.9 ± 6.5%) but not in the Control group (1.5 ± 0.4% versus 2.6 ± 0.7%) **Fig. 3C**. However, in the carotid artery, depletion of NO had no significant effect on the contractile response in either the Obese group (53.0 ± 6.9% vs. 68.5 ± 11.4%) or the Control group (3.3 ± 1.6% vs 2.6 ± 1.4%), **Fig. 3D**.

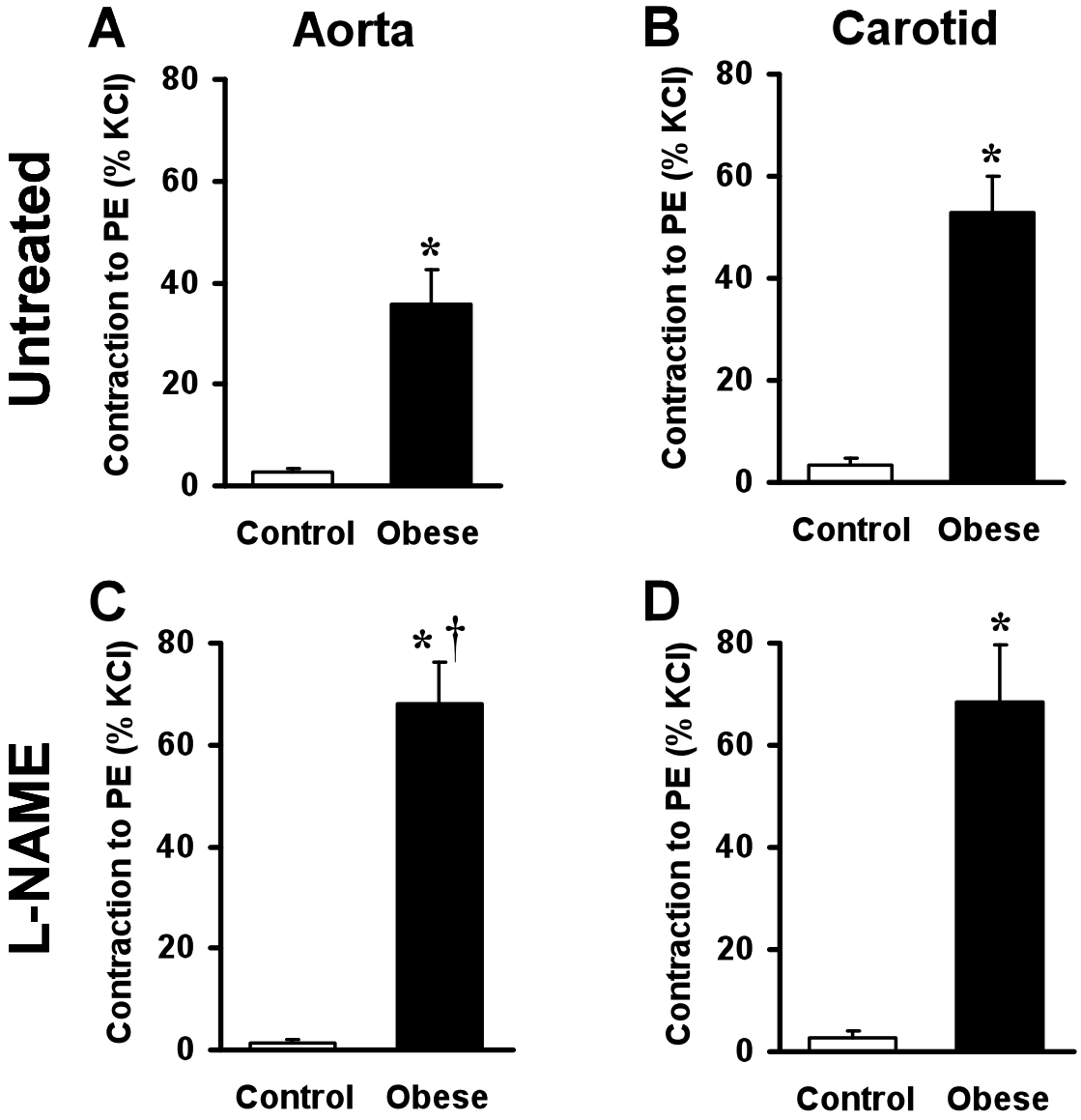

Fig. 3: Contractile responses to PE in aorta and carotid artery rings of mice fed a standard chow (Control) or high-fat diet (Obese) for 30 weeks. Vessels were either left untreated (A and B) or were pretreated with L-NAME (300 μmol/L) for 30 min (C and D) before the exposure to PE (10 nmol/L). Data are expressed as a percentage of KCl. A: Aorta (Control: n=9, Obese: n=7). **P*<0.05 vs. Control. B: Carotid (Control: n=5, Obese: n=7). **P*<0.05 vs. Control. C: Aorta (Control: n=16, Obese: n=8). **P*<0.05 vs. Control, † *P* <0.05 vs. Obese Untreated. D: Carotid (Control: n=11, Obese: n=5). **P*<0.05 vs. Control. Values were compared using the paired or unpaired students *t*-test, as appropriate.

3.3.2. Effect of High-Fat Diet on Response to Acetylcholine

3.3.2.1. Nitric Oxide-Dependent Relaxation to Acetylcholine

Nitric-oxide mediated, endothelium-dependent relaxation to ACh was analyzed in aorta and carotid artery rings after 30 weeks of high-fat diet. Vascular response was first investigated in presence of endoprostanoids (Untreated) as shown in **Fig. 4A** and **B.** In the carotid artery, ACh evoked a biphasic response, **Fig. 4B**, which was not seen in the aorta**, Fig. 4A**. At low concentrations of ACh (<100 nmol/l), carotid artery rings showed a relaxation (maximal relaxation at 30nmol: Control: -83.4 ± 7.1%, Obese: -84.1 ± 4.1%), whereas above this concentration a contractile response was observed (final relaxation: Control: -47.3 ± 7.9%, Obese: -50.2 ± 7.4%), which was not present in aortic rings (final relaxation percentage: Control: -87.9 ± 2.7%, Obese:

-89.0 ± 2.1%), **Fig. 4A**. There was no difference in endothelium-dependent relaxation between Control and Obese groups in either the aorta or the carotid artery. To examine whether prostanoids are involved in the ACh-dependent response, vessels were pretreated with Meclo (1 μmol/L), a non-selective COX inhibitor, **Fig. 4C** and **D**. While relaxation in the aorta remained similar, the concentration-dependent contraction seen in the untreated carotid artery was no longer observed (final relaxation Carotid: Control -97.8 ± 2.2%, Obese -99.7 ± 0.3%; Aorta: Control -92.7 ± 2.1%, Obese -93.0 ± 2.6%), which manifests as significantly different single concentration-values at concentrations of ACh >3 μmol/l for both Carotid Obese and Control groups. When analyzing the AUC (**Table 4**), values differed significantly just between the Obese group of the untreated carotid artery compared with the carotid artery incubated with Meclo. The AUC values of the carotid artery were significantly higher compared with the aorta in the group treated with Meclo, but not in the Untreated groups. The pD2 values for the carotid artery were higher for all groups, indicating that the carotid artery was more sensitive to ACh and reached the half maximal relaxation at a lower concentration.

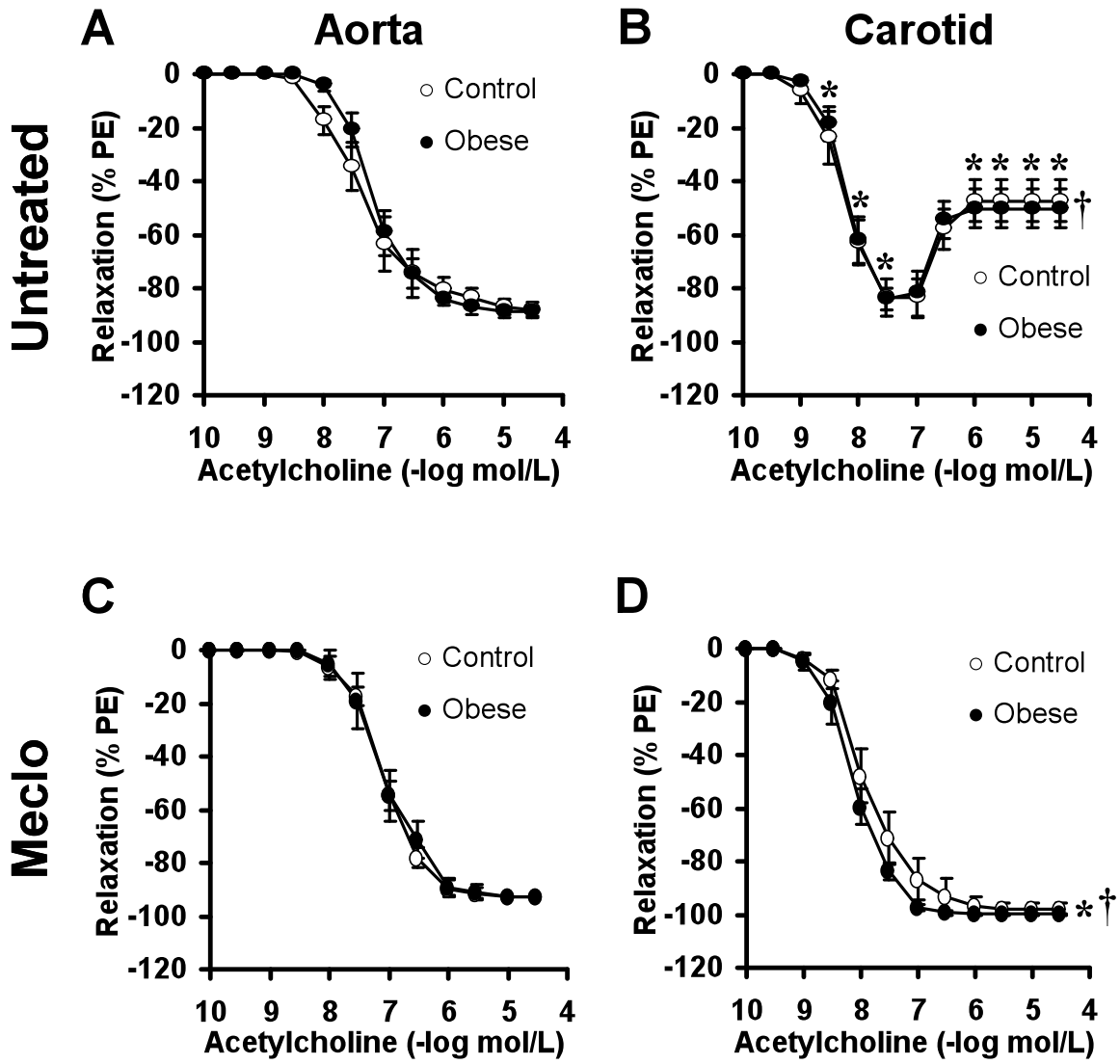

Fig. 4 Endothelium-dependent relaxation to ACh in aorta and carotid artery of mice fed a standard chow (Control) or high-fat diet (Obese) for 30 weeks. Vessels were either left untreated (A and B) or were pretreated with Meclo (1 μmol/L) for 30 min (C and D). Data are expressed as a percentage of precontraction with PE. A: Aorta (Control: n=7, Obese: n=7), B: Carotid artery (Control: n=5, Obese: n=6). **P*<0.05 vs. Aorta Untreated, † *P*<0.05 for pD2 vs. Aorta Untreated (both compared by unpaired students *t*-test). C: Aorta (Control: n=6, Obese: n=6), D: Carotid Artery (Control: n=5, Obese: n=6) **P*<0.05 vs. Aorta Meclo (compared by ANOVA, pD2 and AUC compared by unpaired students *t*-test), † *P*<0.05 for AUC vs. Untreated Obese Carotid, compared by paired students *t*-test. Values for AUC and pD2 are represented below.

Area Under the Curve and Half Maximal Effective Concentration of Responses to Acetylcholine for Vessels left untreated or preincubated with Meclofenamate

		Control		Obese	
Treatment	**Vessel**	**AUC**	**pD2**	**AUC**	**pD2**
Untreated	Aorta	199.7 ± 20.0	7.2 ± 0.2	187.7 ± 12.9	7.1 ± 0.1
	Carotid	218.2 ± 23.0	8.2 ± 0.1*****	214.8 ± 23.9	8.2 ± 0.1*****
Meclofenamate	Aorta	194.0 ± 10.6	7.1 ± 0.1	188.5 ± 18.1	7.1 ± 0.2
	Carotid	279.2 ± 22.4*****	7.9 ± 0.2*****	307.0 ± 10.0***†**	8.1 ± 0.1*****

Table 4: AUC and pD2 values for vascular responses to ACh in the aorta and carotid artery, Control and Obese groups, vessels in presence or absence of Meclo (1 μmol/L). Values represent means ± standard error and are given in arbitrary units for AUC and in mol/l for pD2. * $P<0.05$ vs. Aorta, † $P<0.05$ vs. untreated Carotid. Values were compared by paired or unpaired student's *t*-test where appropriate.

3.3.2.2. Nitric Oxide-Independent Response to Acetylcholine

To examine the response to ACh in NO-depleted conditions, L-NAME was used. The relaxation observed in vessels left untreated or preincubated with meclofenamate (**Fig. 4**) was blocked after pre-treatment with L-NAME (**Fig. 5**). In the aorta, **Fig. 5A,** a contractile response starting at 0.3 μmol/l ACh was seen with no significant difference between the control and obese group (final contraction at 30 μmol/l ACh, Aorta: Control: 14.3 ± 1.9%, Obese: 14.5 ± 1.4%). The carotid artery rings, **Fig. 5B**, were more sensitive and showed a contractile response starting at 0.1μmol/l ACh. Moreover, in these rings, the contractile response differed between the groups and was enhanced in the Obese group as compared with the Control group (final contraction at 30 μmol/l ACh, Carotid: Control 31.7 ± 5.8%, Obese 53.7 ± 3.7%). The AUC values were significantly higher in the carotid artery in comparison with the aorta for both diet groups (**Table 5**). Furthermore, AUC values were higher in the carotid artery from Obese animals as compared to the Control. The pD2 values were higher in the carotid artery, indicating higher sensitivity towards ACh-dependent contraction (**Table 5**).

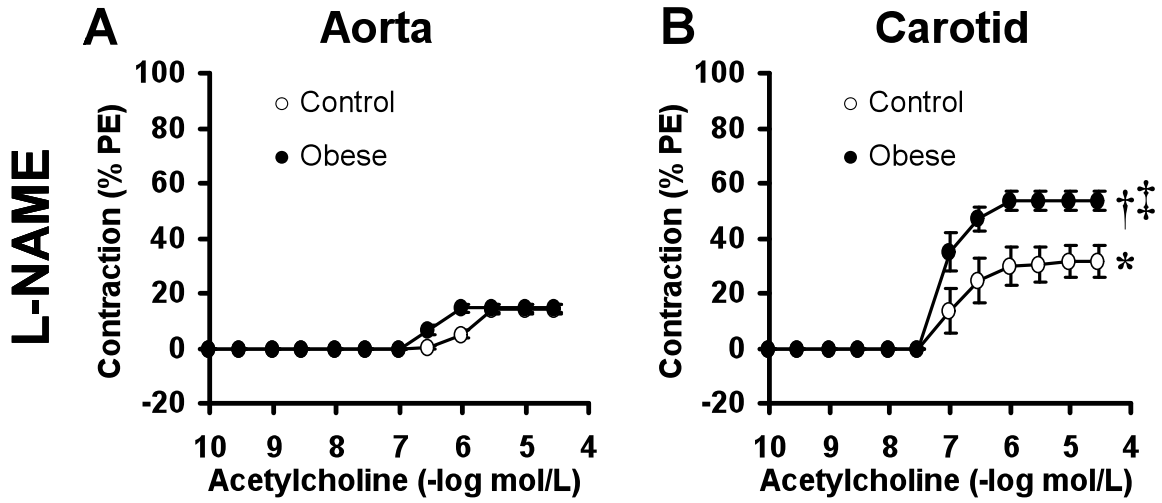

Fig. 5 Endothelium-dependent contraction to ACh in aorta and carotid artery of mice fed standard chow (Control) or high-fat diet (Obese) for 30 weeks. Vessels were pretreated with L-NAME (300 μmol/L) for 30 min. Data are expressed as a percentage of precontraction with KCl. A: Aorta (Control: n=6, Obese: n=7), B: Carotid Artery (Control: n=5, Obese: n=7). *P<0.05 vs. Control Aorta, † P <0.05 vs. Control Carotid Artery, ‡ P <0.05 vs. Obese Aorta, compared by ANOVA.

Area under the curve and half maximal effective concentration of responses to Acetylcholine for vessels preincubated with L-NAME

		Control		Obese	
Treatment	**Vessel**	**AUC**	**pD2**	**AUC**	**pD2**
L-NAME	Aorta	20.5 ± 2.7	6.4 ± 0.04	21.2 ± 2.3	6.5 ± 0.04
	Carotid	56.7 ± 15.4*	6.7 ± 0.2*	106.1 ± 8.6*†	7.0 ± 0.1*

Table 5: AUC and pD2 values for vascular responses to ACh in the aorta and carotid artery after pretreatment with L-NAME (300 μmol/L). Values represent means ± standard error and are given in arbitrary units for AUC and in mol/l for pD2. * *P*<0.05 vs. Aorta, † *P*<0.05 vs. Control Carotid Artery.

4. Discussion

4.1. Summary

The present study was performed to investigate the effect of high dietary fat intake on vascular function in the carotid artery and aorta of young mice. Feeding with high-fat diet increased body weight and led to glucose intolerance and altered vascular tone regulation.

In the thoracic aorta and carotid artery vascular contractions to phenylephrine were increased after high-fat diet. Inhibition of endogenous NO further increased phenylephrine-dependent contractions in aortic rings of the Obese group. Stimulation with ACh evoked endothelium-dependent relaxation. Maximal final values for endothelium-dependent, ACh-mediated relaxation were obtained in prostanoid-depleted conditions, after inhibiting the endogenous prostanoid synthesis using meclofenamate. The values were similar in both groups and remained unaffected by high-fat diet. The carotid artery reacted more sensitively to stimulation with ACh. In native carotid artery rings, high concentrations of ACh induced endothelium-dependent contractions, which were prostanoid-dependent. In the absence of endogenous NO, relaxations to ACh were absent and endothelium-derived contractile responses were observed in both arterial beds instead. These contractions were markedly higher in the carotid artery. Interestingly, high-fat diet further increased endothelium-dependent contractions in the carotid artery, but not in the aorta. Thus, these data suggest that high dietary fat intake induces changes in vascular tone leading to an increased contractile response in elastic type arteries. The heterogenous results in different arteries indicate that the importance of the different pathways regulating the vascular tone varies between vascular beds and is variably influenced by high dietary fat intake.

4.2. Weight Gain and Glucose tolerance

In the present study, mice fed with the high-fat diet were significantly heavier and their glucose tolerance was markedly impaired compared with the control group fed with standard chow. High dietary fat-mediated metabolic changes in C57BL/6J mice are similar to the metabolic syndrome in human beings, since they develop diet-induced obesity and consequent insulin resistance and dyslipidemia.[120-122] These mice are thus an approved rodent model organism for nutritional and vascular research.[123] In the present study, the mice were sacrificed at 34 weeks of age, which, based on their life expectancy (50% survival point is at about 30 months for C57BL/6J mice), relates to early adolescence in human.[124] An important element contributing to the development of obesity is a high caloric diet.[2] The typical "western-style" diet is rich in carbohydrates, especially di- and oligosaccharides, and lipids, containing mainly saturated fatty acids.[123] These diets have been shown to promote overweight, that mainly consists of fat tissue located selectively in the mesentery.[125] As a consequence, metabolic changes like elevated blood pressure,[55] altered insulin synthesis and sensitivity and imbalance of vascular tone regulation occur.[121,126,127] A number of studies have shown that saturated fatty acids have an adverse impact on health.[128] Effects include enhanced lipid storage, a lowered accessibility of the latter once stored and glucose intolerance.[129,130] On the other hand, polyunsaturated fatty acids can have desirable effects.[128] The molecular correlate of these effects still remains elusive. Not only lipids, but also other macronutrients such as protein and carbohydrate may contribute to the development of obesity.[131] A high-fat diet rich in carbohydrates and low on proteins supports fast weight gain, whereas a diet low on carbohydrates and rich in proteins cannot prevent from weight gain, but keeps blood glucose and insulin resistance low.[131] The diets used in this study contains comparable amounts of protein (22.4% kcal of protein for standard chow and 16.4% kcal of protein for high-fat diet), but differs in the composition of lipids (12.3% vs. 58%) and carbohydrates (25.5% vs. 65.4%). The major lipid fraction in the high-fat diet was derived from coconut oil, which mostly contains saturated fatty acids (90%). Thus, besides the

difference of lipid percentage between the diets, the higher intake of saturated fatty acids in the Obese group could have had an effect on changes of vascular responsiveness.

One of the metabolic changes induced by high dietary fat intake is an impaired glucose tolerance. Over the last few years, interactions between glucose- and lipid metabolism and diet have been investigated in detail.[132] Physiologically, in the liver insulin controls the blood glucose levels by activating glucose storage by inhibiting gluconeogenesis and glycogenolysis and activating glyconeogenesis. Several transmembranal glucose transporters are known, some of them are expressed constantly, others (like GLUT4) are induced by hormones like insulin. The expression of the glucose transporters varies between tissues.[133] Besides the expression of glucose carrier molecules, insulin accounts for a variety of regulatory effects in various systems. In the adipose tissue, glucose uptake via the GLUT4 pathway is activated by insulin.[134] Also in the muscle, which accounts for 75% of the stored blood glucose, glucose uptake by GLUT4 transporters is enhanced.[135] Intracellularly, glucose metabolisation into triglycerides is enhanced, but the reverse way (energy mobilisation by lipolysis) is blocked.[136] A high dietary fat intake can influence metabolism by increased body weight and directly via altered insulin action.[129] It is reported that the insulin-dependent translocation of the intracellular localised glucose receptor GLUT4 to the cellular membrane is reduced in type II diabetes.[137] Adiposity is one of the main risk factors for type II diabetes.[138] The latter is pathophysiologically associated with an insulin insensitivity of metabolic organs like the liver, skeletal muscle and the adipose tissue, which leads to glucose intolerance.[139] In the present study, impaired glucose tolerance and greater weight gain was observed in animals fed a high-fat diet. A nuclear receptor involved in this regulation is PPARγ (peroxisome proliferator-activated receptor γ), a lipid sensor molecule modulating gene expression and thereby survival, proliferation and differentiation of different cell types, such as adipocytes.[140] It has been demonstrated that modulation of agonists binding to PPARγ can prevent cells from pathological changes related to obesity and diabetes.[141] Specific deletion of PPARγ in mice adipose tissue diminishes weight gain and glucose intolerance as a consequence of high-fat diet.[142] Oral

antidiabetics of the class of glitazones bind to this receptor in order to decrease insulin resistance, but can also modify synthesis of vascular endothelial growth factor, leptin, interleukins and adiponectin.[141,143,144] Another important player in the regulation of metabolism is the appetite-regulating hormone leptin, which is secreted mainly by fat cells.[145] Obesity induced by high-fat diet leads to leptin-resistance in peripheral tissues[146] and a high saturated-fat diet lowers circulating leptin levels.[147] Interestingly, knockout of cannabinoid receptors lowers leptin and insulin levels similarly, but enhances leptin sensitivity and thus leads to leanness.[148] Beside obesity and diabetes, C57BL/6J mice fed with high-fat diet also develop increased renal lipid accumulation and glomerulosclerosis.[149] High-fat diet can also lead to inflammation of intestinal, adipose and liver tissue.[150] Based on these findings it can be concluded that high-fat diet and obesity do not only interact with the complex system of glucose metabolism and body weight homoeostasis, but can also induce structural changes in organs, tissues and other regulatory mechanisms.

4.3. Effects of High-Fat Diet on Vascular Tone

4.3.1. Phenylephrine-Mediated Contractions

In the present study, vascular contractions to phenylephrine were increased in aorta and carotid arteries after high-fat diet. Possible molecular mechanisms of the increased contractile response to phenylephrine as an effect of high-fat feeding may be: a) change in receptor expression; b) receptor-mediated downstream signalling; c) inhibition of anti-contractile factors. In the aorta, the contractions were further increased by the blockage of endogenous NO synthesis in the Obese group. This suggests that basal NO synthesis is of higher relevance to the balance of contractile and relaxing stimuli in mice fed a high-fat diet.

In the carotid artery, depletion of NO did not further increase PE-mediated contractions, which may be due to: a) involvement of effector molecules other than NO, b) maximal contraction, which cannot be further increased. The findings of the present study are in line with the data of Molnar *et al.*, who fed mice with a high-fat diet for 9 weeks starting at 8 to 10 weeks of age and found

enhanced contractile response in femoral arteries.[151] Reil *et al*., examined the effects of a 26 weeks-high-fat, high-sucrose diet on rat aortas though did not find a change in contractile responses to phenyephrine.[55] A reason for these contradictory findings could be: a) differences in animal species and vascular beds, b) the differences in the constituents of high-fat diet.

In earlier reported studies, obese mice that were fed a high-fat diet showed increased expression levels of vasoconstrictor receptors, leading to various downstream-effects.[21] The animals showed higher activation of the sympathetic nervous system,[127] but reduced adrenergic stimulation of lipolysis due to changed expression of their receptors.[152] One of the contributing changes was an increased ratio of α_2 to β-adrenergic receptors.[83,153] The expression of β_1 and β_3 adrenergic receptors in C57BL/6J mice fed a high-fat diet is reduced and the β-receptor activated adenylyl cyclase activity is diminished in brown and white adipose tissue cells of these mice.[152] Details of the interactions and the downstream receptor signalling still remain elusive, as even though mice lacking all three beta-adrenergic receptors become obese,[154] knock-out mice unable to synthesize norepinephrine and epinephrine (lack of dopamine beta-hydroxylase gene) do not become obese with high-fat diet.[82] It can be summarised that adrenergic agonists have important effects not only on the regulation of vascular tone, but also on the accumulation of body fat and that their receptor expression and distribution can be altered by dietary fat intake.

The findings of the present study indicate that high-fat diet can induce changes in reactivity to adrenergic agents even in young animals.

4.3.2. Nitric Oxide-Dependent Responses to Acetylcholine

In the present study, endothelium-dependent vascular relaxations were examined in the presence of ACh in a concentration-dependent manner. The carotid artery was more sensitive to ACh-mediated relaxation compared to the aorta. The difference may be attributed to: a) the architecture of the vascular ring (e.g. the density of smooth muscle cells), b) the expression level of ACh specific receptor, c) differences in the expression or activity of molecules involved in the intracellular signalling pathway.

Maximal vascular relaxation was similar in the two vascular beds and was unaltered by high-fat diet. These values were obtained by nullifying the influence of endogenous prostanoids by blocking the COX. This finding is contradictory to other studies reporting that vascular relaxation is impaired after high-dietary fat intake. Previously published findings in C57BL/6J mice fed a high-fat diet for 30 weeks had shown a diminished relaxation in the carotid artery of the Obese group.[107] Molnar *et al*. found maximal relaxations of only about 25% to ACh in femoral arteries of mice fed a high-fat diet for 9 weeks (total age 17 to 19 weeks).[151] Reil *et al*., studied rats fed a high-fat, high-sucrose diet for 6 months and found an increase of systemic blood pressure and attenuation of dilatory responses to ACh as well.[55] In the present study, maximal relaxations to ACh of more than 80% were observed in all groups. Several factors may contribute to the differences in ACh-dependent relaxations between the numerous studies: a) different age of the animals, b) differences in the vascular beds, c) differences in dietary fat composition, d) duration of diet, e) the way in which the artery was prepared for the experiment to prevent endothelial injury during the dissection.

In the present study, the initial endothelium-dependent relaxation was followed by a contractile response in the native carotid artery. Treatment with non-selective cyclooxygenase inhibitor meclofenamate abolished these endothelium-dependent contractions, thus cyclooxygenase-derived endoperoxides are probably involved in the endothelium-mediated contractile responses. In NO-depleted conditions after treatment with L-NAME, relaxation was blocked and endothelium-dependent vascular contractions to ACh became more evident in both vascular beds, but varied in

intensity. Carotid artery rings showed a stronger contractile response, which was even more enhanced in the Obese group. In the aorta, no difference between the groups was observed. This suggests that the enhancement of prostaglandin-mediated endothelium-dependent contraction by high-fat diet depends on the vascular bed. Previous studies on high-fat diet have also shown that endothelium-dependent contractions to ACh are enhanced in pathological states like hypertension, diabetes and atherosclerosis in humans.[116,155] Vascular inflammation and reduced NO synthesis can precede peripheral insulin resistance.[156] The dilatory response to ACh is altered by diabetes and the influence of the dilatory effectors decreases.[105] The presence of so-called EDCFs in vascular dysfunction and their dependance on NO synthesis has already been observed by other groups.[103,157] In carotid arteries of ApoE-deficient mice, the synthesis of vasodilating NO is impaired due to lower eNOS-activation in response to hypercholesterolemia.[158] In the mouse artery, COX-1 seems to be involved in mediating endothelium-dependent contraction as inhibition of COX-2 did not block the contractile response.[107] Moreover, the endothelium-dependent contractions are diminished in COX-1 knock-out mice.[106] In other animal models, COX-2 derived prostaglandins have been shown to mediate endothelium-dependent contractions in the aorta.[159]

Furthermore, diet and obesity can alter the synthesis of reactive oxygen species (ROS). Some of these molecules, like the superoxide-anion, have the ability to inactivate vasodilating NO promoting endothelial dysfunction.[160] ROS are suspected to be important mediators of vascular disease.[161] Activation of cholinergic receptors is enhanced in animals fed a high-fat diet. It is coupled to increased superoxide anion production and thus suspected to accout for hypercholesterolemia-induced endothelial dysfunction.[158] High glucose levels and maybe even insulin itself (both found in animals fed a high-fat diet) can contribute to elevated levels of ROS-production.[162] and thus to the development of atherosclerosis.[163]

Antioxidant drugs or superoxide-dismutase-mimetics abolish abnormal vasoconstriction due to cholesterol intake,[109,158] supporting the thesis that oxidation and formation of ROS plays a key role in the development of endothelial dysfunction.[109,164]

Overall, a tendency to vasoconstriction in consequence of high-fat diet can be observed, but changes depend on genetic background, vascular bed and nutritional settings.[21,95,165] Even if there are no macro- or microscopically detectable atherosclerotic lesions in the vascular system, endothelial dysfunction can already be present as a reaction to high dietary fat intake.[166]

4.4. Limitations of the Study and Outlook

In the present study, numbers of animals are in the range of 5 to 16 per group, which signifies limited statistical power. Even with most precaution, mistakes in handling mice, dissecting vessels and preparing chemicals used for the study can never be totally excluded.

To support our findings, it would be interesting to repeat the measurements with higher numbers of mice or to know, if other research groups draw the same conclusions using identical dietary regimens on mice of the same age and strain.

Using tissue of the mice sacrificed for the present study, it would be interesting to investigate the expression profile of ACh and phenylephrine receptors on endothelial cells and vascular smooth muscle cells of arteries. The correlation between the presence of EDCF's in functional studies and the molecular expression profiles could be analyzed.

As only elastic-type arteries were used to investigate the effects of high-fat diet on vascular tone in the present study, it would be interesting to use muscular-type arteries and investigate tissues like skeletal muscle, heart muscle, pancreas or liver for changes on macroscopic, microscopic and molecular level.

As a next step, it would be interesting to investigate the reversibility of diet-induced changes in regulation of vascular tone by returning to normal diet or increasing physical activity after a period of high-fat diet. Furthermore, studies could be conducted on mice of different ages to learn more about the influence of age and vascular remodelling due to high-fat diet.

4.5. Conclusion and Clinical Implication

The present study provides evidence for high-fat diet-induced weight gain, glucose intolerance and endothelial dysfunction in elastic arteries of obese adolescent mice. The disturbed homeostasis of vascular tone manifests as a tendency to vasoconstriction, particularly as an adverse response to ACh, which in healthy vessels causes vasodilation.[157] The influence of high-fat diet, the importance of NO synthesis and the sensitivity to vasoactive molecules vary between different vascular beds. The findings of the present study indicate that NO generally plays a more important role in vessels of mice fed a high-fat diet, since the inhibition of NO-synthesis enhances contractile responses in these animals but not in controls. Moreover, contractions to adrenergic agonists are enhanced in obese animals. Reasons could be a stronger activation of the sympathetic nervous system or changed receptor expression.[127] As a consequence a vicious circle leading to severe dysregulation of vessel tone and thus increased systemic resistance develops.

Endothelium-dependent contractions as a response to ACh similarly occur in humans.[103] Angina pectoris occurs, if the myocardial demand of oxygen exceeds the blood supply through the coronary arteries.[167] Dysregulation of vascular tone can lead to coronary spasms and elicit angina.[168] The spasms can occur at sites of pre-existing stenosis or totally independently. This form of angina is called variant angina pectoris or Prinzmetal-angina. It is a form of angina pectoris which is visible in the echocardiogramm, but is entirely reversible and thus does not lead to structural damage.[169] Smoking clearly promotes variant angina pectoris, but it remains unclear, if the other cardiovascular risk factors have a correlation.[170] In the affected patients, vasospasms can be triggered by activation of muscarinic receptors through exposition to ACh.[171,172] The molecule presumably responsible for the contraction is thus ACh and contractions occur due to pathologic changes in vascular reactivity.[173] It has been hypothesized that the enhanced contraction could be based on a reduced synthesis of NO.[174] Symptoms can be alleviated by treatment with nitrates, which liberate NO or calcium antagonists.[175,176]

Other studies showed that levels of reactive oxygen species (ROS) are higher in obese animals and that ROS degrade NO.[67,177] The important role of reactive oxygen in human vascular disease is supported by the fact that the endothelial function and symptoms of angina are not just improved by treatment with NO-donors, but also with antioxidants.[178] Furthermore, the administration of cyclooxygenase-inhibitors restores levels of NO and thus improves endothelial function, suggesting correlation between enhanced endoprostanoid synthesis and decreased NO levels.[179] There is strong evidence that the contractions to ACh found in endothelial dysfunction are mediated by endogenous prostanoids.[100,180] This is supported by the present study as contractions to ACh found in mice fed a high-fat diet were blocked by inhibition of COX. The contraction to phenylephrine via alpha-adrenergic receptors are endothelium-dependent and PKC is one of the molecules in the intracellular signalling pathway. As mentioned above, contractions to phenylephrine were stronger in mice fed a high-fat diet in the presented study. Interestingly, PKC is upregulated in patients with coronary artery spasms.[181] Mills *et al*. found that mice fed a high-fat diet additionally had a stronger activation of their sympathetic nervous system.[127] Thus, if NO is reduced, ACh paradoxically leads to contractions and adrenergic vasoconstrictor stimuli are enhanced, a tendency to vasoconstriction becomes perspicuous. Obesity due to high dietary fat intake is a widespread disease and its prevalence is still increasing.[5,182] It has been shown that childhood obesity in humans affects cardiac function already at young age and that it increases the risk of cardiovascular morbidity in adulthood and early death.[24,25] The finding of the present study that maximal obtained relaxation is unaltered suggests, that these changes are not of structural nature, but instead are pure regulatory phemonenon. Since it remains unclear, if the observed adverse effects are reversible, reduction of risk factors of the metabolic syndrome in sense of primary and secondary prevention should have first priority. Improvement of vascular function and regression of correlated risk factors can be obtained by dietary restriction and enhanced physical activity.[28,183,184] Interventions should be made especially at young age to lower the risk for cardiovascular morbidity and also to prevent from consecutive cost explosions in the health care sector. If changes have already become symptomatic,

efficient therapies for symptom relieve and prevention from aggravation are needed. More detailed knowledge about altered signalling pathways in diet-induced obesity like the prostanoid, NO, reactive oxygen species synthesis and activity of adrenergic activation could help to improve pharmacological interventions. Modulation of the expression, activation and signalling cascade of receptors could have effects on body weight, the subsequent metabolic changes and development of cardiovascular disease.

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